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PROCESSING NATURAL AND RECONSTITUTED SILK SOLUTIONS UNDER EQUILIBRIUM AND NON-EQUILIBRIUM CONDITIONS

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ABSTRACT

A variety of natural silk secretions (from spiders, silkworms and aquatic insect larvae), and also reconstituted silk solutions, are able to form a nematic liquid crystalline phase. The anisotropic structures that self-assemble in this phase are formed from the isotropic phase by aggregation of molecules, rather than by individual molecules undergoing a conformational change to a rod-like form. This enables the molecules to retain their solubility in water while, simultaneously, the viscosity of the solution is reduced. The liquid crystalline phase is stable under a wide range of equilibrium conditions, but its ability to form *is* sensitive to the rate at which the initially isotropic solution is allowed to dry. The kinetics of phase transitions exhibited by solutions of silk proteins must be taken into account if solutions of silk fibroin are to be successfully processed *in vitro*.

BACKGROUND

Several biosynthesized polymers and their derivatives are useful as engineering materials. While unaltered or transgenic organisms therefore can play an important role as sources of chemical feedstock in materials synthesis, the subsequent processing steps needed to manufacture useful objects are still conducted *in vitro*. There are many benefits of carrying out processing operations (fiber drawing, film forming, injection molding) on polymers in the nematic liquid crystalline state, and of retaining nematic order in the solid product [1-5]. In the case of both liquid crystalline solutions and liquid crystalline melts, these benefits include:

- the low viscosity and therefore easy processability of the liquid crystalline state, compared to isotropic fluids having the same concentration of polymer;
- facilitated production of microstructures in which molecules are extended and globally aligned, leading to enhanced uniaxial stiffness and strength in the solid product;
- the low thermal expansion coefficient of the product;
- reduced susceptibility of the product to retraction (dimensional change arising from randomization of molecular orientational order) when annealed at temperatures above the glass transition but below the melting point;
- the ability to orient non-liquid crystalline materials through guest-host interactions.

In addition, liquid crystalline melts (but not solutions) exhibit relatively low solidification shrinkage compared to conventional melts, and so can be used to obtain moldings with more precise dimensional tolerances. These properties of liquid crystalline polymers are the result of spontaneous local alignment of extended molecules that characterizes the (fluid) liquid crystalline state, reducing the contributions of entanglements to fluid viscosities, and reducing the extent to which molecular re-ordering must occur during solidification.

Examples from all the major classes of biological macromolecule (proteins [6-10], polysaccharides [11-13], glycoproteins [13], nucleic acids [14-16] and lipids [17-19]) are known to form liquid crystalline phases in water. Of particular interest here are macromolecules that form liquid crystalline phases under physiological conditions, so that the liquid crystalline state plays a direct role in the *in vivo* assembly of these molecules into more complex structures, and thus is directly responsible for their function. Then, *in vitro* processing of these materials and their analogs in the liquid crystalline state is an obvious approach towards duplicating the microstructures and properties inspired by the natural materials.

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LIQUID CRYSTALLINITY AND SILK

The silk fibers produced by spiders and the larvae of some insects exhibit a variety of impressive engineering properties; some significant ones are summarized in Table I.

Table I Some Sophisticated Properties of Silk

PROPERTY	COMMENTS	REFERENCE
Mechanical		[20-23]
strength	up to 2 $GN.m^{-2}$ for spider drag line	
stiffness	up to 30 $GN.m^{-2}$ for spider drag line	
toughness	elongation to failure can be greater than 30% for spider drag line	
Processing		[23-25]
solvent	silk fiber is spun from aqueous solution, but will not subsequently dissolve in water (compare Kevlar®, which is spun from a solution in concentrated sulfuric acid)	
processing temperature	silk fiber is spun at ambient temperature	
processing environment	in air or under water	
fiber thickness	fibers may be as fine as 0.01μm	
Environmental		[26, 27]
durability	resists degradation in a wide variety of outdoor and chemical environments	
biodegradability	degradation to the level of amino acids by specific proteolytic enzymes allows spiders to recycle their web silk	

We are especially interested in the *in vivo* processing characteristics of silk secretions. Apart from seeking to reproduce *in vitro* the spinning of fibers from solutions of silk fibroin and similar molecules (regardless of their source), we note the generic usefulness of a room-temperature processing route that converts water-soluble polymer to insoluble high-performance fiber by physical rather than chemical means.

Both as-secreted and reconstituted silk solutions may form a nematic liquid crystalline phase

Qualitatively, we have observed the formation of liquid crystalline order in the silk secretions from diverse organisms and glands:

- silk glands from *Bombyx mori* silkworms;
- drag line, capture thread and cocoon silk secretions from *Nephila clavipes* (golden orb weaver) spiders;
- salivary glands of *Chironomus tentans* (midge) larvae.

Liquid crystalline microstructures of silkworm and spider silk secretions are shown elsewhere. In those cases [10, 28, 29], liquid crystalline microstructures were seen to develop in the viscous secretion recovered by allowing dissected silk glands to leak onto a glass microscope slide.

Microstructures of *Chironomus* silk secretions are shown in Figure 1. Salivary glands were manually dissected from larvae and placed in a droplet of deionized water (@ 20 glands/50 μl droplet) on a siliconized coverslip on the thermostated stage (4 °C) of a dissecting microscope. The lumens were punctured with a needle; secretion was allowed to leak out for about 10 minutes. The glands were removed with dissecting needles, and the extract was transferred to a

microfuge tube and centrifuged at 12,000 x *g*. Supernatants from five extracts were combined; the final sample volume after further concentration was ~50 µl. This solution was initially fluid, compared to undiluted secretion obtained directly from silkworms and spiders. Samples were examined by transmitted polarized light microscopy within 48 hours of preparation. They were allowed to dry slowly between a glass microscope slide and coverslip (Figure 1, left), or rapidly on a microscope slide with no coverslip (Figure 1, right). The phase changes of *Chironomus* silk are of particular interest because the natural silk fibers are produced entirely under water.

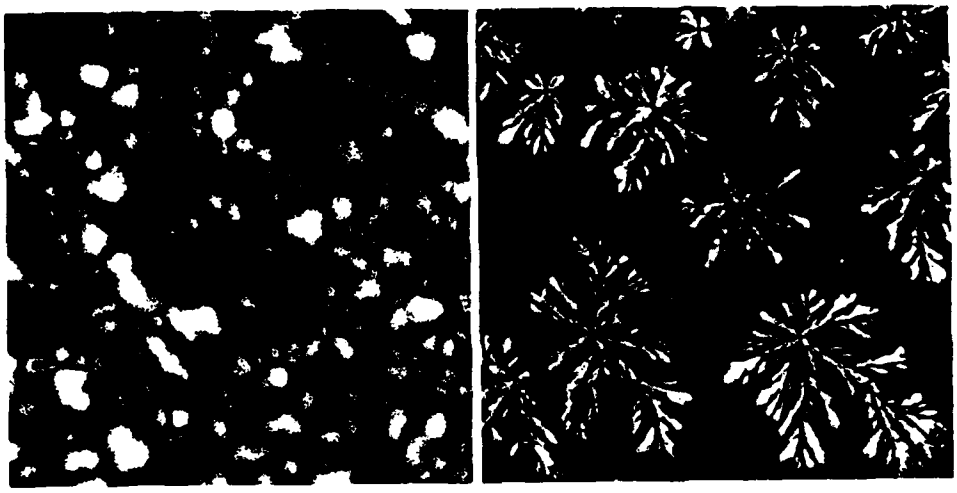

Figure 1 Microstructures of *C. tentans* silk secretion: (left) liquid crystalline order after partial drying under a coverslip; (right) crystalline order after partial drying with no coverslip. Specimens viewed in transmission between crossed polars. 10µm ———

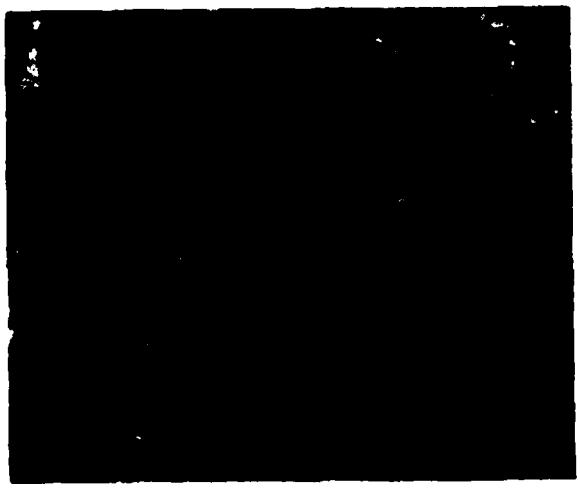

Figure 2 Microstructure of reconstituted *B. mori* silk. Specimen viewed in transmission between crossed polars. 10µm ———

We have also observed that liquid crystalline phases can form in reconstituted silk solutions (Figure 2). The broad range of solution compositions (silk : water : chaotropic salt) over which this occurs has not yet been quantified. The microstructure in Figure 2 is representative of material with the following history: *B. mori* cocoons (with the pupae excised) were boiled in 0.065% NaOH for 2-3 minutes and then in tap water for another 20 minutes, to remove the sericin coating. After drying in a desiccator, silk was dissolved at a concentration of 7.5% by weight in 9.3M LiBr [30]. Approx. 10 grams of the solution were placed in a 10-inch length of cellulose dialysis tubing (Spectra/Por; MWCO 12,000 - 14,000; previously de-sulfurized by

treatment for 30 minutes in 1500 ml water + 30 g sodium bicarbonate + 0.618 g EDTA) and dialyzed against ~200 ml tap water for 2 days.

The liquid crystalline state of silk is assembled from supermolecular anisotropic structures

Several observations imply consistently that the "rods" constituting the liquid crystalline phase of silk are not individual molecules or molecular segments, but instead arise at the supermolecular level:

- silk proteins from different organisms have very different primary structures (none of which is recognizably nematogenic) in solution;
- the phase behavior of silkworm and spider silk secretions implies the existence of rigid rods that have an axial ratio of approximately 30 [13, 29, 31]; however, individual solubilized molecules are flexible and have no significant amounts of secondary structure [32, 33];
- microstructural observations imply that the rods are polydisperse [29].

By aggregating into rod-like structures, silk fibroin molecules with an essentially random coil conformation can form a liquid crystalline phase without having to first undergo a change in conformation. Thus, the fibroin can retain its solubility in water, while at the same time the solution becomes more easily processable because its viscosity is lowered. Fiber spinning is accompanied by a shear-induced conformational change [34]; the molecules then form a stable crystalline structure that is no longer water-soluble. This mechanism may serve as the basis for developing synthetic polymer processing routes that enable the fabrication of durable, water-insoluble fibers from molecules initially in aqueous solution.

Conditions that favor the formation of liquid crystalline order in silk solutions

Because of the experimental difficulties of accurately measuring protein and salt concentration in small samples used for microscopy, reliable quantitative information about the concentration range of liquid crystalline stability is limited. Yet, even qualitative observations show that the nematic phase forms under a wide range of conditions:

- the ability to form a liquid crystalline phase is exhibited by silks of widely differing compositions, i.e it is not sensitive to the sequence of monomers; indeed, in the case of *Chironomus* silk, one is dealing with a mixture of proteins whose apparent molecular weights span two orders of magnitude [25];
- silk can form liquid crystalline phases in substantially different environments – in the natural secreted state (containing only a trace of dissolved components that are not fibroin [22]) as well as in the presence of significant concentrations of LiBr (the material shown in Figure 2 contains LiBr at a concentration of approximately 0.5M);
- in specimens of silkworm and spider silk secretion (initial concentration ~26 vol.% or ~30 wt.% protein) that are maintained between a glass microscope slide and cover slip, a liquid crystalline phase can be recognized within minutes, i.e. after only a small change in concentration due to evaporation; a more ordered phase typically does not occur until after 2-3 days of slow drying.

However, whether or not a continuously drying silk solution exhibits liquid crystalline order does appear to be *sensitive to the initial concentration and the rate at which water is lost.* Fluid solutions, such as water-diluted *Chironomus* silk secretions, initially form liquid crystalline phases if allowed to dry slowly between a glass slide and cover slip. If no cover slip is used, then crystalline phases are formed. Viscous solutions, such as the undiluted silk secretions from silkworms and spiders, form a liquid crystalline phase whether or not a cover slip is present. A similar dependence of microstructure on initial concentration and subsequent drying rate is exhibited by other types of biological macromolecule [13].

REPRESENTING PHASE TRANSITION KINETICS ON TRANSFORMATION DIAGRAMS

Phase diagrams display the phases that occur under equilibrium conditions in a given system. In an open system in which equilibrium is not reached, the phases obtained depend on the driving force for transformation (how far is the system from equilibrium?) and on the extent to which the microstructure is able to rearrange in response to this driving force.

An established representation of the kinetics of temperature-induced phase transformations in multi-component metals and ceramics is by means of Time-Temperature-Transformation (TTT) curves [35]. These curves (Figure 3a) illustrate the time dependence of the microstructural changes exhibited by a system *of fixed composition* at different temperatures. The characteristic "C" shape of the curves results from the fact that, at small supercoolings (close to equilibrium), there is little driving force for the phase change to occur. At large supercoolings, there is significant driving force for the system to convert to the new equilibrium phase, but the atoms or molecules now lack the mobility to achieve this transformation quickly. At intermediate supercoolings, the combination of driving force and mobility enables the transformation to occur at a maximum rate. Typically, TTT curves are drawn for 1% completion and 99% completion of the transformation, though a single curve is often adequate for illustrative purposes. The time difference between 1% completion and 99% completion is most significant for transformations that occur entirely in the solid state.

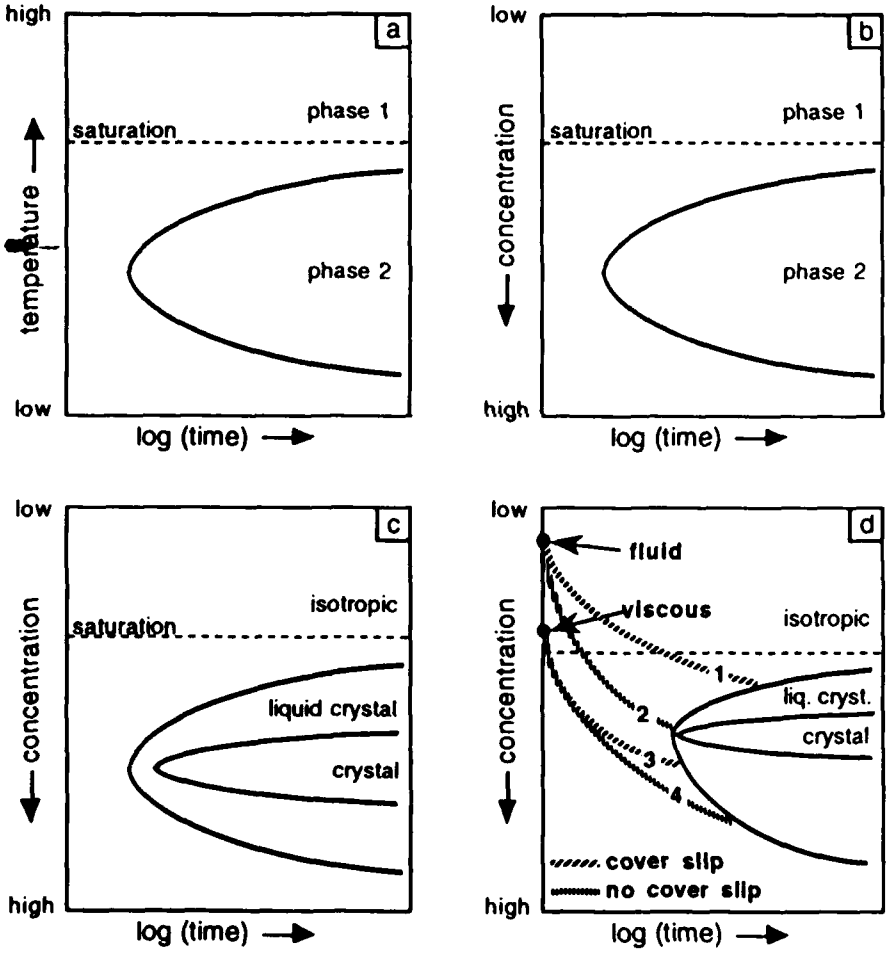

Figure 3 Transformation diagrams showing phase transition kinetics.
(a) Time-Temperature-Transformation relationship for temperature-induced phase changes.
(b) Analogous Time-Concentration-Transformation relationship for concentration-induced phase changes.
(c) Time-Concentration-Transformation curves for a system that forms both liquid crystalline and crystalline phases.
(d) Time-Concentration-Transformation curves schematically representing the behavior of dilute and viscous solutions of silk.

We find that an analogous graphical device provides a useful intellectual framework for illustrating the kinetics of phase transformation in polymer solutions (Figure 3b). For transformations occurring in solutions of different concentration *at a fixed temperature*, the vertical axis is scaled in terms of concentration. The behavior of the system at varying levels of *supersaturation* is shown, rather than at varying degrees of supercooling. Transformations at low supersaturations would occur slowly, due to the system being close to equilibrium, and transformations at very high supersaturations would also occur slowly, since chain interactions would limit microstructural mobility. Again, therefore, a maximum transformation rate would be expected at intermediate supersaturations, where the combination of kinetic drive and molecular mobility would be optimized. Given that the transformation from isotropic material to a mesophase is kinetically easier (requires less microstructural rearrangement) than the precipitation of a three-dimensionally ordered crystalline phase, the transformation curves for mesophase formation and crystallization should be nested as shown (Figure 3c).

The format of these curves is qualitatively consistent with the observed behavior of silk solutions (Figure 3d). Dilute specimens maintained between glass slides (and thus losing water relatively slowly; curve 1) can form liquid crystalline phases, with the possibility of crystallizing at very long times. Dilute specimens without a cover slide will start at the same concentration as those maintained between glass surfaces, but will change in concentration more rapidly. Thus the "drying curve" of the specimen (curve 2) may be able to intercept the "nose" of the crystallization curve. Concentrated specimens with (curve 3) or without (curve 4) a cover slide do not have sufficient microstructural mobility to crystallize immediately after transformation to the liquid crystalline state. The smooth curves (1-4) showing concentration change with time are schematic in the present qualitative discussion; they are discontinuous at the "C" curve for transformation to the liquid crystalline state because the diffusion coefficients of solvent in liquid crystalline and crystalline material will be different.

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